UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON D.C., 20460

OFFICE OF **C**HEMICAL **S**AFETY
AND **P**OLLUTION **P**REVENTION

July 27, 2017

MEMORANDUM

SUBJECT: Protocol Review of an acute *Daphnia magna* toxicity test with NSPW-L30SS

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BACKGROUND

Poly-Technical Solutions submitted a draft protocol for a *Daphnia magna* acute toxicity test with NSPW-L30SS, which contains silver nanoparticles, for agency review. The study was required as part of the registration decision for a conditional registration of NSPW-L30SS[1]. The agency found upon review of the submitted draft protocol that there are several critical components either missing or needing adjustment to address testing and verification of exposure material, and exposure concentrations with a

[1] USEPA. 2015. *Registration Decision for NSPW-L30SS (previously referred to as "Nanosilva"). A Materials Preservative for Use in Textiles and Plastics.* Office of Pesticide Programs, May, 2015. 83 pp. https://www.regulations.gov/document?D=EPA-HQ-OPP-2012-0594-0026

test material containing a metal nanoparticle. The following provides agency recommendations on changes to the study protocol and issues to address in the study protocol that, if addressed appropriately and incorporated, are likely to result in an acceptable *D. magna* acute toxicity study with NSPW-L30SS.

DISCUSSION

The major critical issues with the study include the following areas: lack of inclusion of a paired silver nitrate acute toxicity test with *D. magna*; lack of inclusion of a nanoparticle reference material; need for additional pretest preparation to inform test substance preparation and to inform stability and aging under bioassay conditions; test substance preparation; route of administration; dosing concentrations and test medium; chemical and physical monitoring of a test material with metal nanoparticles; lack of histological sampling and observations; and lack of critical nanomaterial-specific sample preparation and analytical methods. Detailed discussions for each of these is provided in the following bulleted sections. The agency recommends consulting Coleman et al. (2015); Zook et al. (2015); Kennedy et al. (2010, 2017) and USEPA (2009) for modifications to the agency's OCSPP 850.1010 acute *D. magna* toxicity test guideline when testing with a nanomaterial.

- **Include a paired silver nitrate acute toxicity test with *D. magna* in the study.**

The study protocol needs to include paired testing of *D. magna* using a silver salt, such as silver nitrate (recommended salt), which uses the same batch of organisms, dilution water, and environmental conditions with the NSPW-L30SS test. For risk assessment purposes, the toxicity of NSPW-L30SS or the silver particles therein will be compared to conventional silver salt toxicity. Without a paired test, differences in silver toxicity due to factors such as different laboratories, different batch of organisms, different dilution water, especially water hardness and organic carbon content, and environmental conditions may be attributed to the presence of silver nanoparticles, resulting in an overestimate of the toxic potency of NSPW-L30SS. Toxicity of silver salts between and within laboratories have differed by upward of 200 times due to such factors. One of the reasons a daphnid acute toxicity test with NSPW-L30SS was required in the registration decision was to confirm the conclusion that the silver particles in NSPW-L30SS were not significantly more toxic than conventional silver.

- **Include protocols for characterization of test substance**

The test protocol states that NSPW-L30SS will be tested. The reviewer's understanding is that the technical grade of this is an aqueous suspension of nanosilver-silica composite particles with PVP coating. To verify the material and exposure concentration of daphnid to the NSPW-L30SS nanosilver-silica composite material throughout the test, optical and analytical measurements need to be made at several critical points in the study. The study protocol states for the test substance that "The Sponsor assumes responsibility for purity, stability, identity, synthesis methods and location of documentation." For the agency's review of the study's completeness and scientific soundness with a metal nanomaterial, the specific type of information measured on the batch of NSPW-L30SS to be used in the bioassay should be included. It should also be kept in mind that any techniques for fractionating nanosilver particles, nanosilver-silica composite particles and aggregates, the NSPW-L30SS batch would also need to undergo the same process for characterization measurements. Because some filters can result in high adsorption losses, or the filter or act of filtration can cause disequilibria it is critical that pretest preparation include options investigated and results. Measurements should include total, dissolved, and ionic silver concentration, identification and quantification of particles and their size distribution (e.g., silver-silica composite particles, silver nanoparticles, silver chloride particles, silver chloride complexes, etc.). For spiking bioassay test media, the batch of NSPW-L30SS may undergo sonication to disperse loosely agglomerated materials or just be gently mixed by gently inverting a capped container of NSPW-L30SS (Coleman et al., 2015), whichever method is selected this same process should be used before samples are taken from the batch by the Sponsor for characterization. The time between when these measurements

are to be made on the bioassay batch and when the batch of NSPW-L30SS is to be used in the bioassay should also be provided.

- **Correct the test medium hardness and identify that no metal chelators are to be used in the test medium**

The test protocol states that a test medium with a total hardness between 40 and 180 mg/L $CaCO_3$ will be used. This is too large of a stated range and needs to be specified more narrowly. For testing with metals and metal compounds, the test guideline calls for water hardness to be between 40 – 50 mg/L calcium carbonate ($CaCO_3$) (OCSPP 850.1010)[2]. The test guideline also calls for the test species to be *Daphnia pulex* in this case rather than *D. magna* as this water hardness is too soft for acceptable *D. magna* bioassay survival[2]. However, the agency specifically asked for *D. magna* testing for NSPW-L30SS as part of the conditional registration. For testing a metal or metal compound the lower hardness value for testing *D. magna*, 80 mg/L as $CaCO_3$, should be used in this study. This is also the same water hardness used by Kennedy et al. (2010) for the *D. magna* test result the agency used to assess risks for the conditional registration of NSPW-L30SS. Additionally, some reconstituted water recipes call for addition of a metal chelator, such as EDTA. For testing of a metal or metal compounds the test medium used should not include a metal chelator.

- **Test substance preparation: Use a different test substance preparation method**

The use of a water-soluble fraction would not result in an acceptable study for this test material for use by the agency. Toxicity concerns for this metal nanomaterial include interaction and uptake mechanisms with the solid phase that a water soluble fraction approach would not address. Guidance is provided in Coleman, Kennedy, and Harmon (2015) for preparing homogeneous dispersions of working stock solutions from aqueous suspensions of NPs followed by spiking of bioassay media. For toxicological testing, the dispersions are created and used on the same day. Several of these steps include decisions that cannot be made without first doing some pretesting preparation to determine which approach is best. Results of any pretests used to determine the best approach should be provided in the study report to the agency.

- **Test substance preparation and exposure study type: combine study with a kinetic dissolution/stability study in bioassay medium instead of a solubility study**

The protocol identified that the solubility of the test substance in water would be determined prior to testing to determine the need for a water-accommodated fraction approach. However, as the agency identified in the preceding bullet such an approach is not appropriate for this material. What is more critical for an acceptable study is for the agency to understand how the nanosilver-silica composite material and any nonattached nanosilver particles change during the study, and if such changes are impacted within the dilution range of the study. A kinetic dissolution/stability study conducted in the same test medium under the same environmental conditions at representative dilutions would inform whether the test should be conducted as static or static renewal. Additionally, the agency recognizes that the bioeffects of ionic silver, can occur at or below the range of detection under certain environmental conditions, as well as below quantification of nanosilver particles. If this is expected to occur in this test, such a study can help inform what the form of the nanosilver-silca composite and nanosilver particulates are likely to have taken at such a dilution.

[2] https://www.regulations.gov/document?D=EPA-HQ-OPPT-2009-0154-0041, page 7

- **Inclusion of a nanoparticle reference material in the study.**

The protocol needs to include a nanoparticle (NP) reference material such as one available from the National Institute of Standards and Technology (NIST) or NanoComposix (NC). The protocol should state which material was selected, and the basis for the selection. For example, for an aqueous-based suspension of NPs the NC citrate coated 30 nm silver or NIST RM 8012 citrate-stabilized 30 nm gold aqueous-based NP standards could be used. NP reference material results are used to validate the preparation and protocols followed by the testing facility for creating a working stock solution of NPs and spiking working stock NP suspension into aqueous phase bioassay media.

- **Dose verification and sample handling and processing**

The protocol does not describe the type of measurements that will be made to verify exposure concentrations nor were the sample handling and analytical methods identified other than standard methods but methods appropriate for identifying and quantifying nanomaterials need to be included. The protocol needs to identify specific analytical methods and approaches for determination of total, dissolved, and ionic silver concentration, identification and quantification of particles and their size distribution (e.g., silver-silica composite particles, silver nanoparticles, silver chloride particles, silver complexes, etc.). Additionally, a measure of the distribution of particles in the test container needs to be made. Because it is possible that bioeffects will occur in some test concentrations below quantification limits of ionic silver and/or nanosilver particles methods, alternative analytical approaches that allow quantification should be considered. Additional test containers for each treatment may be needed to increase sample volume. Historically "dissolved" or "soluble" concentration has been based on operational definitions such as: (1) the concentration of chemical retained in the supernatant of a conventionally centrifuged sample of test media[3,4] or (2) the concentration of chemical retained in the supernatant after being passed through a 0.45 µm filter[4,5]. Both of these operational definitions may include some colloidal material not removed by centrifugation or filtration, respectively. Because conventional silver toxicity is expressed in terms of "dissolved" silver it is important that such a measurement is included.

- **Include histological sampling and observations**

The protocol needs to include collection of histological samples and observations. One of the concerns for the fate of nanomaterials is the difference in uptake and distribution within organisms as compared to ionic silver. Organisms should be sampled at the end of the study from the control, and from the highest NSPW-L30SS concentration for which organisms survived, the same for the silver nitrate reference. A portion of the animals that died during the study should also be examined.

Other recommended changes:

- A.3. Methods Guidelines. Should indicate the guidelines or references followed for modifications made to address testing with a metal nanomaterial.

[3] USEPA. 1994. Pesticide Reregistration Rejection Rate Analysis. Ecological Effects. Special Review and Reregistration Division and Environmental Fate and Effects Division, Office of Pesticide Programs. 738R94035. Available at www.nepis.epa.gov.

[4] USEPA. 2016. OCSPP 850.1000 Background and Special Considerations – Tests with Aquatic and Sediment-Dwelling Fauna and Aquatic Microcosms. Office of Chemical Safety and Pollution Prevention. EPA 712-C-16-014. 53 pp. Available as EPA-HQ-OPPT-2009-0154-0042 at www.regulations.gov .

[5] SW-846 Test Method 3005A: Acid Digestion of Waters for Total Recoverable or Dissolved Metals for Analysis by FLAA or ICP Spectroscopy. Available at https://www.epa.gov/hw-sw846/sw-846-test-method-3005a-acid-digestion-waters-total-recoverable-or-dissolved-metals .

- B.3. Contaminants. A discussion of what trace metal clean laboratory techniques or procedures are to be employed to minimize introduction of silver and dust particulates which could impact results should be added. Silver is a compound that is toxic in the part per trillion to part per billion range and may potentially be introduced into the laboratory environment and test media from different sources (e.g., some air purifiers have silver, cross-contamination from handling of silver jewelry, pool filters, antismoking lozenges, etc.). Similarly, certain optical counts of particles can be impacted by introduction of dust particles.

- B.5. Add exposure study type: static, static-renewal or flow through and basis for choice.

- B.5.b. Because this is a metal or metal compound, report the total hardness, and magnesium, calcium, sodium, potassium, iron, copper, zinc, and total organic carbon concentration in test bioassay medium and the highest treatment concentration at test initiation.

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